H2S Exposure Induces Autophagy Injury in The Jejunum of Broilers Through miR-181a-PKC-δ Axis Mediated ER Stress

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Research

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Abstract

Background: Hydrogen sulfide (H_2S), as a toxic atmospheric and industrial pollutant, is one of the main components of haze. Air exposure to H_2S can cause multiple organ and tissue damage. However, studies on the animal intestines of H_2S toxicity are limited. To clarify the effect of H_2S exposure on intestinal proteomics and the role of autophagy in intestinal injury, in the present study, effects of H_2S exposure on jejunum damage of broilers were examined by establishing a 42-day H_2S exposure broiler model.

Results: We observed the morphological changes that arose, screened differentially expressed proteins (DEP_S) and enriched related pathways via electron microscopy, proteomics and bioinformatics analyses, qRT-PCR, and western blot. We obtained 86 proteins that were significantly different under H_2S exposure, including proteins related to autophagy and ER stress regulation. Meanwhile, protein-transcriptomics combined analysis found that autophagy and ER stress-associated pathways MAPK signaling pathway, FOXO signaling pathway, mTOR signaling pathway and Autophagy were significantly enriched. Besides, the expression of the autophagy-related indicators LC3II/I, Dynein, Beclin1, BNIP3, and ULK1 were remarkably up-regulated under H_2S exposure ($P < 0.05$). In contrast, the activities of p62 and mTOR were significantly decreased ($P < 0.05$).

Conclusions: The above results indicate that H_2S induced autophagy in the jejunum via the miR-181a-PKC-δ axis activates ER stress to induce p38/JNK expression. In our study, the combined analysis of proteomics and transcriptomics on broiler intestines was described for the first time and verified the selected pathways related to ER stress and autophagy. These results provide novel insights into the toxicology of H_2S and environmental research.

Highlights

- H_2S exposure triggers 55 DEPs up-regulation and 31 DEPs down-regulation in the broiler jejunum.
- H_2S exposure activates p38/JNK pathway through the miR-181a-PKC-δ axis, increasing autophagy.
- H_2S exposure triggers ER stress pathway via the miR-181a-PKC-δ axis, inducing autophagy.

Introduction

Hydrogen sulfide (H_2S) has become one of the main features of air pollution with the development of industry and agriculture. As a well-known environmental and industrial pollutant, H_2S is one of the main components of smog. It is mainly derived from volcanic eruption (Heggie, 2009), human activities and sources such as the natural gas industry, garbage landfills and sewage treatment plants (Yin et al., 2020). Meanwhile, it has been reported that in modern poultry farms, H_2S in poultry houses is one of the most dangerous air pollutants in the by-products of fecal decomposition (Wang et al., 2011). As a highly toxic and odorous gas, the highest average concentration of H_2S in the environment is about 0.5-8ppb per hour, which is related to landfill odor and poses a severe threat to public health in the United States and other

countries (Heaney et al., 2011). However, air pollution in the animal living environments poses a threat, and potentially damages other aspects of animal welfare. For example, with the intensification of the poultry industry, H_2S is a main harmful gas, affecting the health of poultry and workers in poultry farms (Ni et al., 2020). Meanwhile, H_2S is detrimental to the respiratory system, digestive system and nervous system of humans and other animals (Chen et al., 2019). Studies have shown that acute exposure to high concentrations of H_2S could cause severe brain damage, and long-term neurological disorders (Kim et al., 2016). Even exposure to low levels of H_2S in a short time poses a health threat, as it is rapidly absorbed into the lungs and causes blood poisoning (Carpenter et al., 2017). Excessive concentration of H_2S in poultry house not only affect the production performance of poultry, but also cause trachea necrosis, respiratory system damage, inflammation, oxidative stress and immune system damage in broilers (Li et al., 2019). In addition, energy metabolism disturbance induced by inhalation of H_2S is associated with LPS mediated apoptosis. The intestine is a target organ of a variety of air pollutants (Guo et al., 2019). Intestinal tissue is the target organ of various air pollution. It has been reported that H_2S exposure induced the activation of CYP450 and oxidative stress in the jejunum of broilers, which promoted oxidative stress and caused jejunum tissue damage (Zheng et al., 2019).

MicroRNA (miRNA) is a kind of noncoding RNA composed of 21-25 nucleotides that participates in gene expression regulation through base-pairing with the complementary mRNA 3'UTR sequence. MiRNAs have become a focus of toxicology research. For example, a study found that under the influence of H_2S, miR-393 targeted TIR1 to improve organism resistance (Shi et al., 2015). Besides, miRNAs play important roles in regulating autophagy. The study found that miR-181a induced essential in rat cardiomyocytes, which resulted in cardiac hypertrophy (Li et al., 2017). Autophagy may be an important pathway of cell death caused by H_2S (Wang et al., 2017). The development of autophagy is complex and accompanied by a variety of upstream regulatory signaling pathways. PRKCD (PKC-δ) is an important factor in regulating autophagy, and its expression can induce autophagy (Lin et al., 2012). PKC-δ is also associated with ER stress, which generates a stress response in colon cancer cells, eventually causing cell death (Lim et al., 2008). Furthermore, ER stress also activates the expression of p38/JNK (Mishra et al., 2018), which has been reported to promote FOXO1 activity(Fang et al., 2019; Yan et al., 2017). FOXO1 has been shown to activate autophagy through the mTOR and BNIP3 signaling pathways and thereby increase the activities of LC3II/I and Dynein but decrease the activity of p62. A large number of studies show that LC3 is a marker of autophagy, with increases in LC3 indicating the occurrence of autophagy (Gong et al., 2019; Zhuang et al., 2019). The above observations show that PKC-δ induces autophagy through the mTOR and BNIP3 signaling pathways under various of conditions.

Nowadays, proteomics, as an effective bioinformatics analysis method, has been applied more and more in toxicology. Liu et al. used TMT-based quantitative proteomics to find that H_2S caused inflammation and cell death in pig lung tissue through IL-17 and other signaling pathways (Liu et al., 2020). Furthermore, Using proteomics, Ryzhikov et al. revealed how inflammation affected the temporal structure of autophagy in the mouse liver (Ryzhikov et al., 2019). The previous research in our lab found that H_2S exposure caused significant changes in the jejunum transcriptome. However, the autophagy

damage of H_2S exposure in the jejunum of broilers and the mechanism of H_2S-induced autophagy are unclear. This study aimed to evaluate the pathological process with the participation in autophagy of jejunum injury, to identify the jejunum proteomic changes, and to preliminary explain the mechanism of intestinal autophagy induced by H_2S. Therefore, we chose to construct the model of H_2S exposure in broilers, observed the ultrastructural changes of jejunum after H_2S exposure by the transmission electron microscope, and detected differentially expressed proteins (DEPs) in jejunum by proteomics and principal component analysis (PCA). GO (Gene Ontology) enrichment, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway performed to screen out signal transduction pathways significantly affected by H_2S exposure, and to predict the targeted regulation of miR-181a and PKC-δ. The expression of miR-181a, PKC-δ, JNK/p38, and autophagy-related indicators under H_2S exposure were detected by qRT-PCR and western blot. Our study to clarify the effects of H_2S exposure on broiler jejunum proteomics. It provides a new understanding of H_2S toxicology and the foundation for comparative medicine.

Materials And Methods

Experimental animal model

All procedures performed in this study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (SRM-11). Eighty (1-day-old) male and female (1:1) broilers (Weiwei Co., Ltd., Harbin China) were divided into two groups at random: a control group and an H_2S group. There were 4 parallel subgroups in each group, with 10 broilers per subgroup. In the control group, broilers were raised under normal conditions, without exposure to H_2S gas. The room was filled with anhydrous H_2S gas for 24 hours for 0-3 weeks in the H_2S group. For 4-6 weeks, anhydrous H_2S concentration was maintained between 19.5mg/kg and 20.5mg/kg. All broilers were fed the same diet and water and housed in two independent environmental control rooms that differed only in the injection of anhydrous H_2S gas. At the age of 42 days, 15 broilers were randomly selected for euthanasia. Jejunum tissues of broilers were collected, immediately frozen in liquid nitrogen, and stored at -80℃ for further experiments.

Electron microscopic observation of jejunum in broilers

The jejunum tissue samples were fixed in 2.5% glutaraldehyde and 1% osmium solution, dehydrated with an alcohol gradient stained with 4.8% uranyl acetate, and dried in the environment without moisture. Then, ultrathin sections stained with uranyl acetate were used for observation via transmission electron microscopy (GEM-1200ES, Japan).

Protein expression analysis

A total of 0.5mL protein extraction buffer (50 mM Tris-HCl, 4% SDS-PAGE, 100 mM NaCl, pH 8.0) was added to each sample for protein extraction. The protein concentrations in the supernatant were then analyzed using the BCA method. Proteomics analysis was done by the Proteomics Research Center of Beijing Institute of Animal Science and Veterinary Medicine, Chinese Academy of Agricultural Sciences.

Next, 100μg of proteins were subjected to trypsin digestion and labeling of different peptides. The mixed labeled peptides were separated by high pH reversed-phase liquid chromatography with an Agilent 1290 high-pressure liquid chromatography system. Then, 2% acetonitrile + 0.1% formic acid water was used to dissolve each polypeptide sample fully. The analyses were carried out on the Easy-NLC 1000 system (Thermo Scientific) and Orbitrap Fusion Tribrid MS liquid chromatography-mass spectrometry system. Then, the proteomes UP000002494 from the UniProt Proteomes database was searched for mass spectrometry data using Maxquant (vision 1.6.4). Finally, quality control analysis was carried out, including the evaluations of protein enzyme cutting effect and the accuracy and stability of mass spectrometry results.

Difference factors enrichment, network, and pathway analyses

Complete transcriptomics data (Zheng et al., 2019) and proteomics data were used to identify data differences, screen out GO terms and apparent signal pathways enriched by differentially expressed factors through MetaboAnalyst 5.0, GO terms in database for functional annotation, KEGG database, DAVID (version 6.8) and KOBAS databases. In the Metascape (A Gene Annotation & Analysis Resource) and OmicShare (https://www.omicshare.com/), GO terms network and KEGG network help us determine the signal pathways involved in this study. Using Cytoscape and STRING (version 11.0), we conducted the interaction networks of the selected difference factors.

Quantitative real-time PCR (qRT-PCR)

Extraction of total RNA from the jejunum of broilers by the TRIzol method and transcribed them into cDNA for further study as previously described (Wang et al., 2018). qRT-PCR was performed using SYBR® Premix Ex Taq™ (Takara, Beijing, China) with the Light Cycler® 480 system (Roche, Basel, Switzerland). We selected β-actin as an internal reference, and each sample was divided into three parallel groups. We designed primer sequences (**Table 1)** using Primer Premier software 5.0 (Premier Biosoft International, USA). The data were analyzed by the $2^{-\Delta\Delta t}$ calculation method.

Western blot analysis

Western blot analysis was used to detect protein expression following previously described procedures (Cui et al., 2019). The primary antibodies included antibodies against PKC-δ (1:500), GRP78 (1:1500), XBP1 (1:1000), FOXO1 (1:500), ULK1 (1:500), p38 (1:500) and JNK (1:500, Wanleibio Co., Ltd., Liaoning, China), Beclin1 (1:1000), p62 (1: 500), mTOR (1:200), Dynein (1:500) and LC3 (1:100, produced by our lab), BNIP3 (1:500), β-actin (1:1000) and peroxidase-conjugated antibody against rabbit IgG (1:500, Santa Cruz, CA, USA) was used as a secondary antibody. The developer was added to analyze the protein.

Statistical analysis

The data analysis was conducted using t-tests analysis with IBM SPSS for Windows (version 23; SPSS Inc., Chicago, IL, USA). All the data were normally distributed, and the results are presented as the mean ±

standard deviation (SD). '*' represents a statistically significant difference ($P < 0.05$) between the control group and the H_2S group.

Results

Morphological examination of broiler jejunum

To evaluate the effect of H_2S exposure on autophagy in the jejunum of broilers, the ultrastructural changes of intestinal tissues were observed by transmission electron microscopy. In the control group (**Fig. 1A**), continuous and complete cell membrane, endoplasmic reticulum, and mitochondrial structure were observed in the intestinal tissue cells. A large number of jejunum cells in the H_2S exposure group showed cristae rupture and swelling in the mitochondrion, nuclear atrophy, reduced endoplasmic reticula (indicated by green and blue arrows in **Figure 1b**), and autophagosomes increased significantly (indicated by red arrow in **Figure 1b**). These findings clearly showed H_2S exposure resulted in autophagy of broiler jejunum cells.

Results of proteomic research, and analysis of biological pathways integrated proteomics and transcriptomics data

To clarify the effect of exogenous H_2S exposure on the proteomics of jejunum tissues, we used the TMT isotope method to detect jejunum tissue. Initially, we conducted PCA on the control group data and the H_2S group data to show the data's validity. **Figure 2A** showed the 2D Score Plot in the PCA of proteomics. It can be seen from the figure that the main separation direction of the control group and the H_2S group (there are three replicates in each group). The separation between the two groups was obvious, indicating a significant difference in the data expression. Afterward, we can further screen and analyze the data. 8746 proteins were screened according to FDR < 0.01. After a homology search of the identified protein sequences, quantitative proteome analysis was performed. The number of protein groups identified with this project was 3053, among which 2874 satisfied screening criteria. DEPs were identified according to a threshold P-value < 0.05 and $|\log_2 FC| > 0.585$. **Fig. 2B** showed a heat map of 86 DEPs, 55 DEPs of which were up-regulated and 31 DEPs were down-regulated between the control group and the H_2S group. To classify DEP_S functionally, all DEP_S of the H_2S group and control group were labeled with GO terms. GO terms divide the function of DEPs into three ontologies: molecular function (MF), cellular component (CC), gene and biological process (BP). Using the GO database can identify the DEPs for the enrichment of function analysis (**AdditionalTable 1**). **Figure 2C** showed the GO heat map related to autophagy on proteomics. For cellular components, the major areas of classification were peroxisomal matrix, cotranslational protein targeting to membrane, and establishment of protein localization to endoplasmic reticulum. For biological processes, the major areas of classification were protein targeting to ER, alternative mRNA splicing via spliceosome and collagen metabolic process. For molecular functions, the major areas of classification were collagen binding and protein complex binding. These results suggested that the occurrence of changes in protein metabolism and transport after H_2S exposure.

Additionally, we used the KEGG pathway database for significance enrichment analysis to obtain the most important biochemical metabolic pathways and signal transduction pathways in which DEPs may be involved. The significantly enriched pathways identified in the KEGG pathway enrichment analysis are shown in **Fig. 2D**, and pathways related to autophagy such as protein export of pathway have changed significantly.

In order to better analyze the proteomics results, we performed an integrating analysis of proteomics and transcriptomics. The Venn diagram of **Figure 2E** represented a total of 9 factors in proteomics and transcriptomics with significant differential expression. Most of these factors were involved in the occurrence of ER stress and autophagy. Among them, PKC-δ was significantly expressed and closely related to the expression of other differential factors. Next, GO functional annotation and KEGG signal pathway enrichment analysis were performed on the selected differential factors. The relationship between GO terms was shown in **Figure 2F**, indicating that protein processing in ER was related to ER stress. In addition, **Figure 2G** showed that the network diagram of KEGG significantly enriched pathways such as MAPK signaling pathway, FOXO signaling pathway, mTOR signaling pathway and Autophagy, and they were closely related. Based on the above results, a gene interaction network diagram was used to reveal the interaction between differentially regulated factors in the pathway (**Figure 2H**). Among them, green globules represent the up-regulated genes, and light red globules represent the downregulated genes. The combined analysis showed that the critical factors related to H_2S exposure were related to autophagy and ER stress. The results of protein interaction analysis using STRING for selected proteins are shown in **Figure 2I**. The selected factors related to autophagy and ER stress are also closely related to the protein level.

Detection of miR-181a and PKC-δ in the jejunum tissues of broilers

The biological prediction website TargetScan (http://www.targetscan.org/vert/) predicted that miR-181a and PKC-δ have a targeted relationship in different animals, as shown in **Fig. 3A**. Therefore, to assess the relationship between miR-181a and PKC-δ after H_2S exposure, qRT-PCR was used to detect their expression in the jejunum of broilers. The expression of miR-181a **(Fig. 3B)** exhibited a trend opposite of PKC-δ **(Fig. 3C)**. Additionally, the level of protein expression of PKC-δ, shown in **Fig. 3D**, was consistent with the mRNA expression level determined by qRT-PCR. These results suggested that the targeting relationship between miR-181a and PKC-δ was regulated by H_2S.

mRNA and protein expression of ER stress related indicators in jejunum tissues of broilers

It is necessary to estimate ER stress in jejunum cells of broilers exposed to H_2S. We further studied the mRNA expression levels of ER stress-related genes, XBP1 and GRP78 in broilers exposed to H_2S increased significantly ($P < 0.05$), as shown in **Fig. 4A**. However, the protein expression levels of XBP1 and GRP78 **(Fig. 4B)** were consistent with the results of qRT-PCR. The results showed that ER stress had occurred in the jejunum tissues of broilers exposed to H_2S.

mRNA and protein expression of JNK, p38 and FOXO1 in jejunum tissues of broilers

To verify the effect of upstream pathway factors on autophagy after H_2S exposure, we analyzed the mRNA and protein expression of genes related to the activation of the autophagy pathway (JNK, p38 and FoxO1), as shown in **Figure 5**. The mRNA and protein levels of JNK, p38 and FOXO1 were significantly up-regulated in the jejunum of broilers under the condition of H_2S exposure ($P < 0.05$). Among the proteins, FOXO1 showed the highest increase in protein expression in the H_2S group relative to the control group.

mRNA and protein expression of autophagy-related indicators in jejunum tissues of broilers

To further evaluate the effect of H_2S exposure on the autophagy-related signaling pathways in the jejunum of broilers, we detected autophagy-related indicators. The results of qRT-PCR showed that the mRNA levels of Beclin1, LC3, BNIP3, ULK1 and Dynein were significantly increased ($P < 0.05$), however, those of p62 and mTOR were decreased ($P < 0.05$) in the H_2S group relative to the control group (**Fig. 6A)**. The expression level of LC3II/I, a critical marker of autophagy, significantly increased in the H_2S group relative to the control group ($P < 0.05$). Western blot analysis was used to determine the expression of Beclin1, Dynein, mTOR, BNIP3, ULK1, LC3, and p62 in the jejunum of broilers. Under H_2S exposure, the protein levels of autophagy-related indicators were increased, whereas those of p62 and mTOR were decreased ($P < 0.05$) in **Fig. 6B**. These results indicated that autophagy occurred in the jejunum of broilers exposed to H_2S.

Discussion

Kim et al. identified the acute neurodegeneration of cells induced by H_2S and the molecular mechanisms by analyzing the key molecules and the molecular pathways in the broad-spectrum proteome (Kim et al., 2018). Similarly, it has been reported that DEPs associated with autophagy death pathway by using proteomic analysis increased significantly in AS-6 treated cells, therefore, which was induced by ER stress (Kang et al., 2012). Lindberg et al. studied the skin sensitization effects of glyphosate, the surfactant polyethoxylated tallow amine (POEA) and two commercial glyphosate-containing formulations in human dendritic cells (DC) - like cell lines by using multiple omics techniques (transcriptional and proteomic analysis) (Lindberg et al., 2020). On the premise that PCA proved that there were significant differences between the control group and the H_2S group. The results of our study showed that all 86 DEPs in the jejunum of broilers exposed to H_2S, 55 DEPs of which were up-regulated and 31 DEPs were down-regulated. The most obvious change of expression through comparative analysis was in the proteins related to autophagy and ER stress. The classification, location, function and pathways of DEPs were analyzed by the bioinformatics methods of GO and KEGG. We finally analyze the molecular mechanism after H_2S exposure. GO terms were mainly associated with protein targeting to ER, extracellular exosome, extracellular vesicle, extracellular organelle and peroxisomal part, which were primarily related to autophagy and ER stress. KEGG pathway analysis of altered proteins showed that signal pathways such as protein export played a vital role in the autophagy reaction. Our previous study analyzed the

transcriptome changes in broiler jejunum under H_2S exposure. Therefore, in this study, we proposed a comprehensive analysis of proteomics and transcriptomics. The results showed that 9 different factors were significantly expressed in omics, and PKC-δ was closely related to the occurrence of autophagy (Song et al., 2008). Notably, elements in GO annotations showed that protein processing in the endoplasmic reticulum was involved in the occurrence of ER stress, and autophagy were developed by ER stress through protein targeting to ER (Miao et al., 2020). It is worth noting that KEGG analysis showed that different factors were significantly enriched in the MAPK signaling pathway, FOXO signaling pathway, mTOR signaling pathway and Autophagy, and these pathways were involved in the regulation of autophagy (Thiyagarajan et al., 2016). These results confirmed our previous hypothesis that H_2S triggers ER stress in broiler jejunum tissues and further explained the molecular mechanism of autophagy.

H_2S exposure causes changes in miRNA expression. In previous studies showed miR-20 has involved in ammonia-induced intestinal apoptosis in broiler jejunum through the joint analysis of transcriptomics and ceRNA hypothesis (Wang et al., 2020). Similarly, in our study, the expression level of miR-181a decreased under H_2S exposure. miR-181a is a kind of miRNA associated related to the regulation of autophagy. In SGC7901/CDDP cells, inhibiting the expression level of miR-181a could lead to autophagy (Zhao et al., 2016). PKC-δ-dependent signaling pathway is also an essential mechanism for inducing autophagy in cells (Kumar et al., 2016). Chen et al. confirmed that miR-181a has a targeting relationship with PKC-δ in human cervical cancer cells (Chen et al., 2014). Meanwhile, the analysis of the TargetScan website confirmed that PKC-δ and miR-181a in broilers were highly conserved in rats, cows, horses and other animals. And miR-181a and PKC-δ also have the same binding site. Our results demonstrated that H_2S exposure inhibited miR-181a and the expression of PKC-δ increased, confirming that H_2S exposure caused changes in the miR-181a-PKC-δ axis in the jejunum of broilers. ER stress is closely related to PKC-δ, and it develops following the formation of the PKC-δ-AP-l complex occurs through PKC-δ binding of AP-l in the ER (Qi et al., 2008). ER stress is the feature by the increase of GRP78 and XBP1 (Gundamaraju et al., 2019). Evidences suggest that ER stress also increases the level of FOXO1 expression (Kim et al., 2019). It has been reported that p38 and JNK were activated by PKC-δ in the kidney proximal tubular cells of mice (Pabla et al., 2011). Additionally, the level of FOXO1 expression was found to be regulated by p38 and JNK in LPS-stimulated RAW264.7 cells (Wang et al., 2018). Li et al. found that p38/JNK activated the expression of FOXO1 in primary human aortic endothelial cells (Li et al., 2009). Our results also indicated that H_2S exposure activated PKC-δ at the mRNA and protein levels. In contrast, the expression of ER stress-related proteins XBP1, GRP78 and p38/JNK and FOXO1 were significantly up-regulated, indicating that H_2S exposure activated p38/JNK/by up-regulating PKC-δ FOXO1 axis. FOXO1 plays an important role in autophagy (Kishino et al., 2017), inducing autophagy and cell death through the BNIP3 and mTOR pathways (Wang et al., 2018; Valenzuela et al., 2015). In this study, we observed that the increase in FOXO1 expression activated the BNIP3 and mTOR pathways and that the autophagy marker LC3II was activated after H_2S exposure. In MCF-7 human breast cancer cells, miR-181a increased the expression of LC3 by inhibiting the level of mTOR, which stimulated autophagy (Tekirdag et al., 2013). Chen et al. reported that the increased level of Beclin1 expression through PKC-δ/JNK1 signals transduction

mechanism, which further promoted autophagy (Chen et al., 2009). Our experimental results indicated that H_2S induced the occurrence of autophagy in jejunum tissues by influencing the miR-181a-PKC-δ axis, which led to autophagy by stimulating autophagy-related indicators through the BNIP3 and mTOR signaling pathways.

Conclusion

In summary, the results of our research provided evidence that under H_2S exposure 86 DEPs were significantly expressed in the broiler jejunum through proteomics analysis. The combined analysis of proteomics and transcriptomics successfully annotated the factors into the related pathways of ER stress and autophagy. PKC-δ is one of the key factors for differential expression under H_2S exposure. It is driven by miR-181a, causing ER stress and increasing the expression level of p38/JNK. In addition, the expression of autophagy-related indexes LC3II/I, Dynein, Beclin1, BNIP3 and ULK1 were up-regulated, while p62 and mTOR activities were down-regulated. These findings indicate that H_2S exposure activates ER stress through miR-181a targets PKC-δ, promoting autophagy in the jejunum tissue. The present study enriches the mechanisms underlying toxic in the jejunum tissues of broilers under H_2S exposure and provides insight into the diagnosis and treatment of H_2S poisoning.

Abbreviations

H_2S: Hydrogen sulfide; DEPs: Differentially expressed proteins; PCA: principal component analysis; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MF: Molecular function; CC: Cellular component; BP: Gene and biological process

Declarations

Ethics approval and consent to participate

All procedures performed in this study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (SRM-11).

Consent for publication

Not applicable.

Availability of data and material

All data generated and analyzed during this study are included in this published article.

Competing interests

We declare that they have no known competing economic interests or personal relationships with other persons or organizations that could have appeared to affect the work reported in this paper.

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Authors' contributions

WJZ carried out the experiment, analyzed and interpreted the data regarding the proteomics analysis, and was a major contributor in writing the manuscript. JMG contributed to some methods proposing. TC contributed to the software application. SFZ and SWX conceptualized review, editing the manuscript, and supervised and correct the data. All authors read and approved the final manuscript.

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References

1. Kishino A, Hayashi K, Hidai C, Masuda T, Nomura Y, Oshima T. XBP1-FoxO1 interaction regulates ER stress-induced autophagy in auditory cells. Scientific reports 2017; 7: 4442, 10.1038/s41598-017-02960-1.
2. Wang C, Liu E, Li W, Cui J, Li T. MiR-3188 Inhibits Non-small Cell Lung Cancer Cell Proliferation Through FOXO1-Mediated mTOR-p-PI3K/AKT-c-JUN Signaling Pathway. Frontiers in pharmacology 2018; 9: 1362, 10.3389/fphar.2018.01362.
3. Valenzuela CA, Escobar D, Perez L, Zuloaga R, Estrada JM, Mercado L, et al. Transcriptional dynamics of immune, growth and stress related genes in skeletal muscle of the fine flounder (Paralichthys adpersus) during different nutritional statuses. Developmental and comparative immunology 2015; 53: 145-57, 10.1016/j.dci.2015.06.007.
4. Carpenter Thomas S., Rosolina Samuel M., Xue Zi-Ling. Quantitative, colorimetric paper probe for hydrogen sulfide gas. Sensors and Actuators B: Chemical 2017; 253: 846-851, 10.1016/j.snb.2017.06.114.
5. Chen Jl, Lin Hh, Kim Kj, Lin A, Ou Jh, Ann Dk. PKC delta signaling: a dual role in regulating hypoxic stress-induced autophagy and apoptosis. Autophagy 2009; 5: 244-6, 10.4161/auto.5.2.7549.
6. Chen M, Li X, Shi Q, Zhang Z, Xu S. Hydrogen sulfide exposure triggers chicken trachea inflammatory injury through oxidative stress-mediated FOS/IL8 signaling. Journal of hazardous materials 2019; 368: 243-254, 10.1016/j.jhazmat.2019.01.054.
7. Chen Y, Ke G, Han D, Liang S, Yang G, Wu X. MicroRNA-181a enhances the chemoresistance of human cervical squamous cell carcinoma to cisplatin by targeting PRKCD. Experimental cell research 2014; 320: 12-20, 10.1016/j.yexcr.2013.10.014.

8. Cui Y, Yin K, Gong Y, Qu Y, Liu H, Lin H. Atrazine induces necroptosis by miR-181-5p targeting inflammation and glycometabolism in carp lymphocytes. Fish & shellfish immunology 2019; 94: 730-738, 10.1016/j.fsi.2019.09.068.
9. Kim DH, Ha S, Choi YJ, Dong HH, Yu BP, Chung HY. Altered FoxO1 and PPARγ interaction in age-related ER stress-induced hepatic steatosis. Aging 2019; 11: 4125-4144, 10.18632/aging.102042.
10. Fang P, Sun Y, Gu X, Shi M, Bo P, Zhang Z, et al. Baicalin ameliorates hepatic insulin resistance and gluconeogenic activity through inhibition of p38 MAPK/PGC-1α pathway. Phytomedicine : international journal of phytotherapy and phytopharmacology 2019; 64: 153074, 10.1016/j.phymed.2019.153074.
11. Gong Zg, Wang Xy, Wang Jh, Fan Rf, Wang L. Trehalose prevents cadmium-induced hepatotoxicity by blocking Nrf2 pathway, restoring autophagy and inhibiting apoptosis. Journal of inorganic biochemistry 2019; 192: 62-71, 10.1016/j.jinorgbio.2018.12.008.
12. Guo J., Xing H., Chen M., Wang W., Zhang H., Xu S. H2S inhalation-induced energy metabolism disturbance is involved in LPS mediated hepatocyte apoptosis through mitochondrial pathway. Sci Total Environ 2019; 663: 380-386, 10.1016/j.scitotenv.2019.01.360.
13. Heaney Cd, Wing S, Campbell Rl, Caldwell D, Hopkins B, Richardson D, et al. Relation between malodor, ambient hydrogen sulfide, and health in a community bordering a landfill. Environmental research 2011; 111: 847-52, 10.1016/j.envres.2011.05.021.
14. Heggie Tw. Geotourism and volcanoes: health hazards facing tourists at volcanic and geothermal destinations. Travel medicine and infectious disease 2009; 7: 257-61, 10.1016/j.tmaid.2009.06.002.
15. Tekirdag KA, Korkmaz G, Ozturk DG, Agami R, Gozuacik D. MIR181A regulates starvation- and rapamycin-induced autophagy through targeting of ATG5. Autophagy 2013; 9: 374-85, 10.4161/auto.23117.
16. Kang Jh, Chang Yc, Maurizi Mr. 4-O-carboxymethyl ascochlorin causes ER stress and induced autophagy in human hepatocellular carcinoma cells. The Journal of biological chemistry 2012; 287: 15661-71, 10.1074/jbc.M112.358473.
17. Kim Dongsuk, Anantharam Poojya V., Whitley Elizabeth M., Gilbreath Ebony, Mahama Belinda Y., Kanthasamy Arthi, et al. Acute Exposure to High Level of Hydrogen Sulfide Induces Neurodegeneration via Activation of Inflammasomes in Brain. Faseb Journal 2016; 30,
18. Kim Ds, Anantharam P, Hoffmann A, Meade Ml, Grobe N, Gearhart Jm, et al. Broad spectrum proteomics analysis of the inferior colliculus following acute hydrogen sulfide exposure. Toxicology and applied pharmacology 2018; 355: 28-42, 10.1016/j.taap.2018.06.001.
19. Kumar R, Sahu Sk, Kumar M, Jana K, Gupta P, Gupta Ud, et al. MicroRNA 17-5p regulates autophagy in Mycobacterium tuberculosis-infected macrophages by targeting Mcl-1 and STAT3. Cellular microbiology 2016; 18: 679-91, 10.1111/cmi.12540.
20. Wang LC, Wei WH, Zhang XW, Liu D, Zeng KW, Tu PF. An Integrated Proteomics and Bioinformatics Approach Reveals the Anti-inflammatory Mechanism of Carnosic Acid. Frontiers in pharmacology 2018; 9: 370, 10.3389/fphar.2018.00370.

21. Li Al, Lv Jb, Gao L. MiR-181a mediates Ang II-induced myocardial hypertrophy by mediating autophagy. European review for medical and pharmacological sciences 2017; 21: 5462-5470, 10.26355/eurrev_201712_13936.
22. Li X, Rong Y, Zhang M, Wang Xl, Lemaire Sa, Coselli Js, et al. Up-regulation of thioredoxin interacting protein (Txnip) by p38 MAPK and FOXO1 contributes to the impaired thioredoxin activity and increased ROS in glucose-treated endothelial cells. Biochemical and biophysical research communications 2009; 381: 660-5, 10.1016/j.bbrc.2009.02.132.
23. Li X., Chen M., Shi Q., Zhang H., Xu S. Hydrogen sulfide exposure induces apoptosis and necroptosis through lncRNA3037/miR-15a/BCL2-A20 signaling in broiler trachea. Sci Total Environ 2019; 699: 134296, 10.1016/j.scitotenv.2019.134296.
24. Lim Jh, Park Jw, Kim Sh, Choi Yh, Choi Ks, Kwon Tk. Rottlerin induces pro-apoptotic endoplasmic reticulum stress through the protein kinase C-delta-independent pathway in human colon cancer cells. Apoptosis : an international journal on programmed cell death 2008; 13: 1378-85, 10.1007/s10495-008-0264-z.
25. Lin M, Chandramani-Shivalingappa P, Jin H, Ghosh A, Anantharam V, Ali S, et al. Methamphetamine-induced neurotoxicity linked to ubiquitin-proteasome system dysfunction and autophagy-related changes that can be modulated by protein kinase C delta in dopaminergic neuronal cells. Neuroscience 2012; 210: 308-32, 10.1016/j.neuroscience.2012.03.004.
26. Lindberg T, De vila Ri, Zeller Ks, Levander F, Eriksson D, Chawade A, et al. An integrated transcriptomic- and proteomic-based approach to evaluate the human skin sensitization potential of glyphosate and its commercial agrochemical formulations. Journal of proteomics 2020; 217: 103647, 10.1016/j.jprot.2020.103647.
27. Liu Z, Fu Q, Tang S, Xie Y, Meng Q, Tang X, et al. Proteomics analysis of lung reveals inflammation and cell death induced by atmospheric HS exposure in pig. Environmental research 2020; 191: 110204, 10.1016/j.envres.2020.110204.
28. Miao G, Zhang Y, Chen D, Zhang H. The ER-Localized Transmembrane Protein TMEM39A/SUSR2 Regulates Autophagy by Controlling the Trafficking of the PtdIns(4)P Phosphatase SAC1. Molecular cell 2020; 77: 618-632.e5, 10.1016/j.molcel.2019.10.035.
29. Mishra Rr, Belder N, Ansari Sa, Kayhan M, Bal H, Raza U, et al. Reactivation of cAMP Pathway by PDE4D Inhibition Represents a Novel Druggable Axis for Overcoming Tamoxifen Resistance in ER-positive Breast Cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 2018; 24: 1987-2001, 10.1158/1078-0432.Ccr-17-2776.
30. Pabla N, Dong G, Jiang M, Huang S, Kumar MV, Messing RO, et al. Inhibition of PKCδ reduces cisplatin-induced nephrotoxicity without blocking chemotherapeutic efficacy in mouse models of cancer. The Journal of clinical investigation 2011; 121: 2709-22, 10.1172/JCI45586.
31. Ni Jq, Erasmus Ma, Croney Cc, Li C, Li Y. A critical review of advancement in scientific research on food animal welfare-related air pollution. Journal of hazardous materials 2020: 124468, 10.1016/j.jhazmat.2020.124468.

32. Gundamaraju R, Vemuri R, Chong WC, Bulmer AC, Eri R,. Bilirubin Attenuates ER Stress-Mediated Inflammation, Escalates Apoptosis and Reduces Proliferation in the LS174T Colonic Epithelial Cell Line. International journal of medical sciences 2019; 16: 135-144, 10.7150/ijms.29134.
33. Ryzhikov M, Ehlers A, Steinberg D, Xie W, Oberlander E, Brown S, et al. Diurnal Rhythms Spatially and Temporally Organize Autophagy. Cell reports 2019; 26: 1880-1892.e6, 10.1016/j.celrep.2019.01.072.
34. Shi H, Ye T, Han N, Bian H, Liu X, Chan Z. Hydrogen sulfide regulates abiotic stress tolerance and biotic stress resistance in Arabidopsis. Journal of integrative plant biology 2015; 57: 628-40, 10.1111/jipb.12302.
35. Song Ks, Kim Js, Yun Ej, Kim Yr, Seo Ks, Park Jh, et al. Rottlerin induces autophagy and apoptotic cell death through a PKC-delta-independent pathway in HT1080 human fibrosarcoma cells: the protective role of autophagy in apoptosis. Autophagy 2008; 4: 650-8, 10.4161/auto.6057.
36. Thiyagarajan V, Sivalingam Ks, Viswanadha Vp, Weng Cf. 16-hydroxy-cleroda-3,13-dien-16,15-olide induced glioma cell autophagy via ROS generation and activation of p38 MAPK and ERK-1/2. Environmental toxicology and pharmacology 2016; 45: 202-11, 10.1016/j.etap.2016.06.005.
37. Wang S, Wang W, Li X, Zhao X, Wang Y, Zhang H, et al. Cooperative application of transcriptomics and ceRNA hypothesis: LncRNA-107052630/miR-205a/G0S2 crosstalk is involved in ammonia-induced intestinal apoptotic injury in chicken. Journal of hazardous materials 2020; 396: 122605, 10.1016/j.jhazmat.2020.122605.
38. Wang Ss, Chen Yh, Chen N, Wang Lj, Chen Dx, Weng Hl, et al. Hydrogen sulfide promotes autophagy of hepatocellular carcinoma cells through the PI3K/Akt/mTOR signaling pathway. Cell death & disease 2017; 8: e2688, 10.1038/cddis.2017.18.
39. Wang W, Chen M, Jin X, Li X, Yang Z, Lin H, et al. HS induces Th1/Th2 imbalance with triggered NF-κB pathway to exacerbate LPS-induce chicken pneumonia response. Chemosphere 2018; 208: 241-246, 10.1016/j.chemosphere.2018.05.152.
40. Wang Y, Huang M, Meng Q, Wang Y. Effects of atmospheric hydrogen sulfide concentration on growth and meat quality in broiler chickens. Poultry science 2011; 90: 2409-14, 10.3382/ps.2011-01387.
41. Qi X, Mochly-Rosen D. The PKCdelta -Abl complex communicates ER stress to the mitochondria - an essential step in subsequent apoptosis. Journal of cell science 2008; 121: 804-13, 10.1242/jcs.024653.
42. Yan F, Chen Y, Azat R, Zheng X. Caenorhabditis elegansMulberry Anthocyanin Extract Ameliorates Oxidative Damage in HepG2 Cells and Prolongs the Lifespan of through MAPK and Nrf2 Pathways. Oxidative medicine and cellular longevity 2017; 2017: 7956158, 10.1155/2017/7956158.
43. Yin K, Cui Y, Qu Y, Zhang J, Zhang H, Lin H. Hydrogen sulfide upregulates miR-16-5p targeting PiK3R1 and RAF1 to inhibit neutrophil extracellular trap formation in chickens. Ecotoxicology and environmental safety 2020; 194: 110412, 10.1016/j.ecoenv.2020.110412.
44. Zhao J, Nie Y, Wang H, Lin Y. MiR-181a suppresses autophagy and sensitizes gastric cancer cells to cisplatin. Gene 2016; 576: 828-33, 10.1016/j.gene.2015.11.013.

45. Zheng S, Jin X, Chen M, Shi Q, Zhang H, Xu S. Hydrogen sulfide exposure induces jejunum injury via CYP450s/ROS pathway in broilers. Chemosphere 2019; 214: 25-34, 10.1016/j.chemosphere.2018.09.002.
46. Zhuang J, Nie G, Yang F, Cao H, Xing C, Dai X, et al. Molybdenum and Cadmium co-induced the levels of autophagy-related genes via adenosine 5'-monophosphate-activated protein kinase/mammalian target of rapamycin signaling pathway in Shaoxing Duck (Anas platyrhyncha) kidney. Poultry science 2019; 98: 6533-6541, 10.3382/ps/pez477.

Tables

Table 1 Primer sequences involved in this experiment were used for quantitative real-time PCR (qRT-PCR)

Gene	Forward primer (5□-3□)	Reverse primer (5□-3□)
β-actin	CCGCTCTATGAAGGCTACGC	CTCTCGGCTGTGGTGGTGAA
LC3-□	TTACACCCATATCAGATTCTTG	ATTCCAACCTGTCCCTCA
LC3-□	AGTGAAGTGTAGCAGGATGA	AAGCCTTGTGAACGAGAT
Beclin1	CGACTGGAGCAGGAAGAAG	TCTGAGCATAACGCATCTGG
JNK	CAGATAAGCAGTTAGATGAGAG	GACAGATGACGACGAAGAT
PKC-δ	TCTGTCCTGGCGGAGCGTTG	GCACTGCCATCAGCACCTTC
XBP1	TCAGAGCGCCCGCGACAG	GCAGCAGCCGTTGGTTCTCC
GRP78	GCGGGCGGACGATGAGGAG	GTAGGATGGCGTGATGCGG
ULK1	ACAGACCACGAGGACCAGGATG	AAGTGCCTGCCAGTGAATG
FOXO1	AACGCCTGGGGTAACCTGTCC	GCACGCTCTTGACCATCCAC
BNIP3	TCAGCCCGCAGGAGGAGAAC	CCACGCTGTTTCCATTGCCA
p38	GCATCAACACTCCGCTCCCTAAC	GGCTGTCTTGTCGTAGGCAT
p62	GCTGATGCAGTGGAGGAAGTAGAG	GGAAGCACAGATCGGCTGGAAG
Dynein	AATCAGTGAGCCCACCCAGT	AGCACCCAGAACCGAATC T
mTOR	GGACTCTTCCCTGCTGGCTAA	TACGGGTGCCCTGGTTCTG
U6	CACGCAAATTCGTGAAGCGTTC	
miR-181a	AACAUUCAACGCUGUCGGUGAGU	

Figures

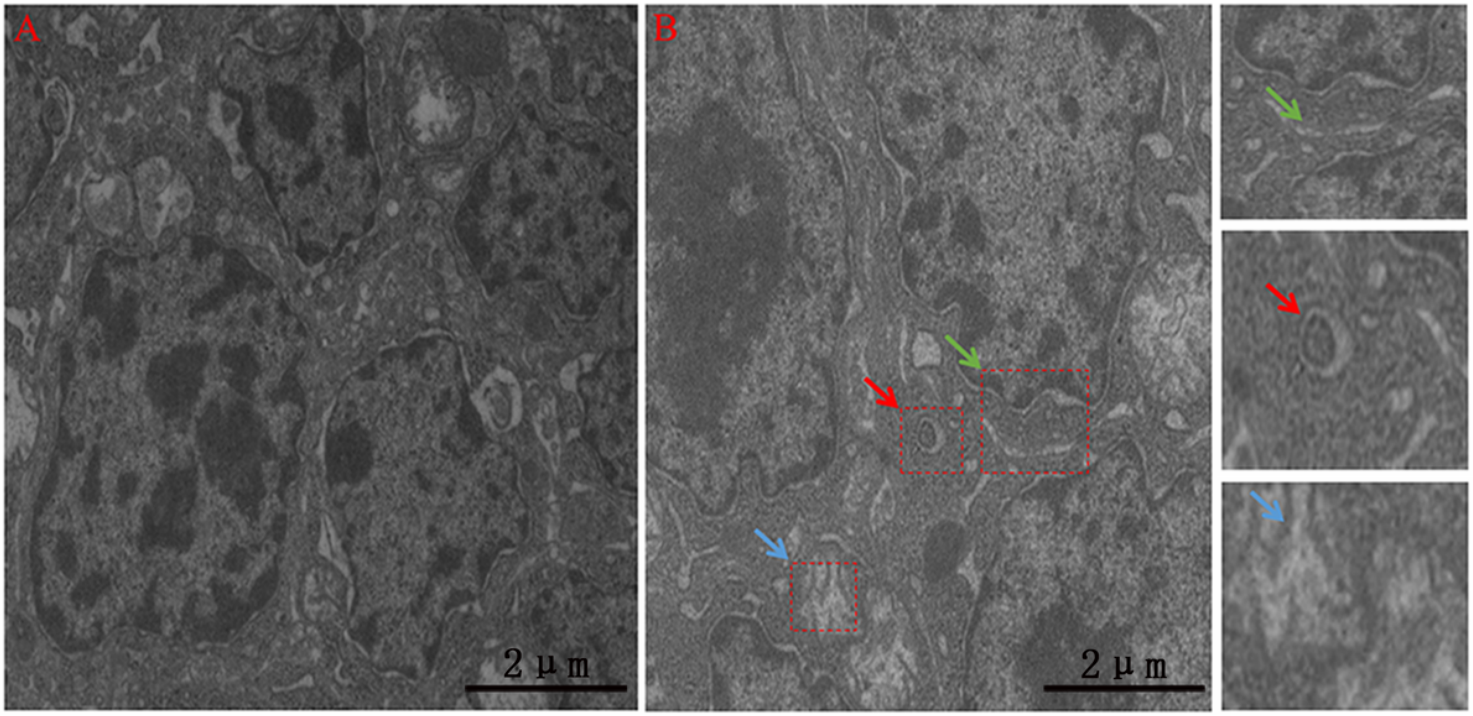

Figure 1

Ultrastructural changes of broiler jejunum exposed to H2S. There were significant differences in the jejunum's ultrastructure between the control group (A) and the H2S group (B). Magnification 8000x. In the H2S group, autophagic vesicles (red arrow), mitochondrial damage (blue arrow) and ER stress (green arrow) were evident.

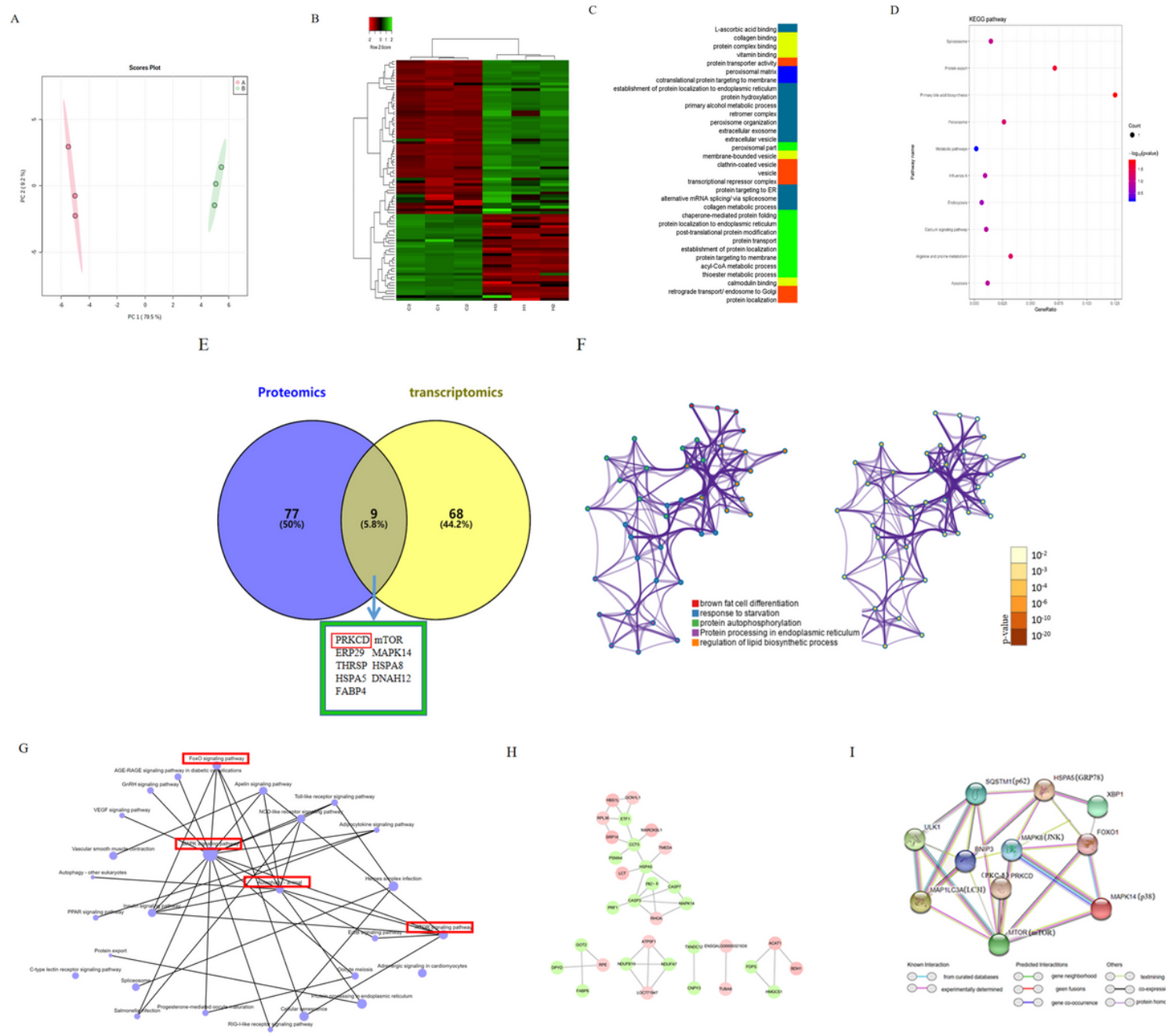

Figure 2

The results of proteomic research and joint analysis of proteomics and transcriptomics. (A) Principal Component Analysis of proteomics. (B) Heat map of DEPs selected by bioinformatics analysis in the group exposed to H2S. (C) Heat map of enrichment of GO terms related to autophagy. (D) KEGG pathway analysis of proteins enriched in the jejunum in the H2S group of broilers. (E) Venn diagram of proteomics and transcriptomics combined analysis. (F) GO interaction network relationship. (G) KEGG signaling pathway interaction network. (H) DEPs network analysis. (E) Relationships among several important proteins in the pathway from String. Different colors correspond to different interaction types.

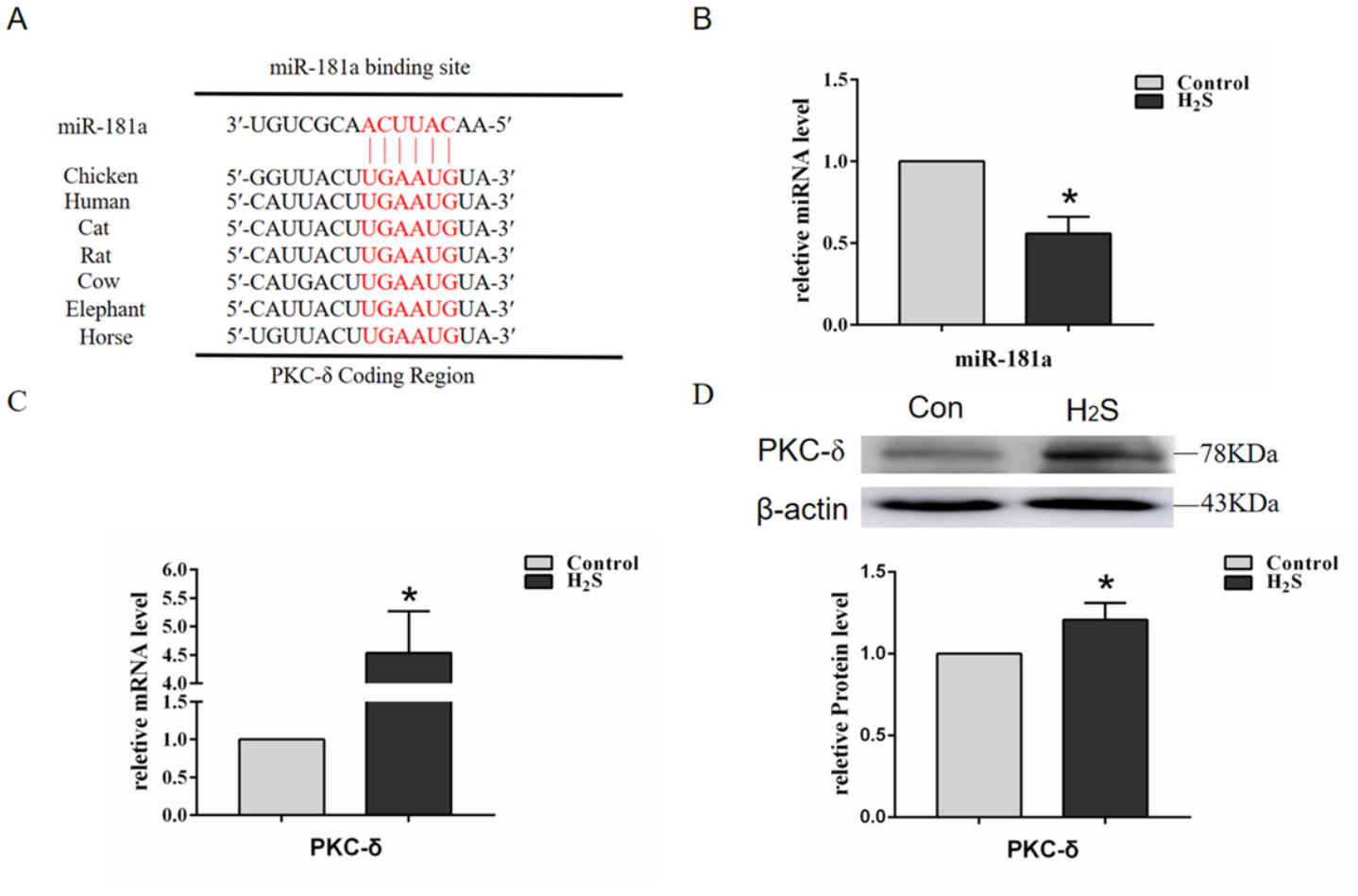

Figure 3

Expression levels of miR-181a and PKC-δ in broiler jejunum under H2S exposure. (A) The TargetScan database was used to predict the binding sites for miR-181a and the target site in the 3′ UTR of PKC-δ from different animals. (B) miRNA expression of miR-181a in the jejunum of broilers. (C, D) mRNA and protein levels of PKC-δ. Data represent the mean ± SD (n = 3). '*' above the bars denotes a significant difference ($P < 0.05$) between the control group and the H2S group.

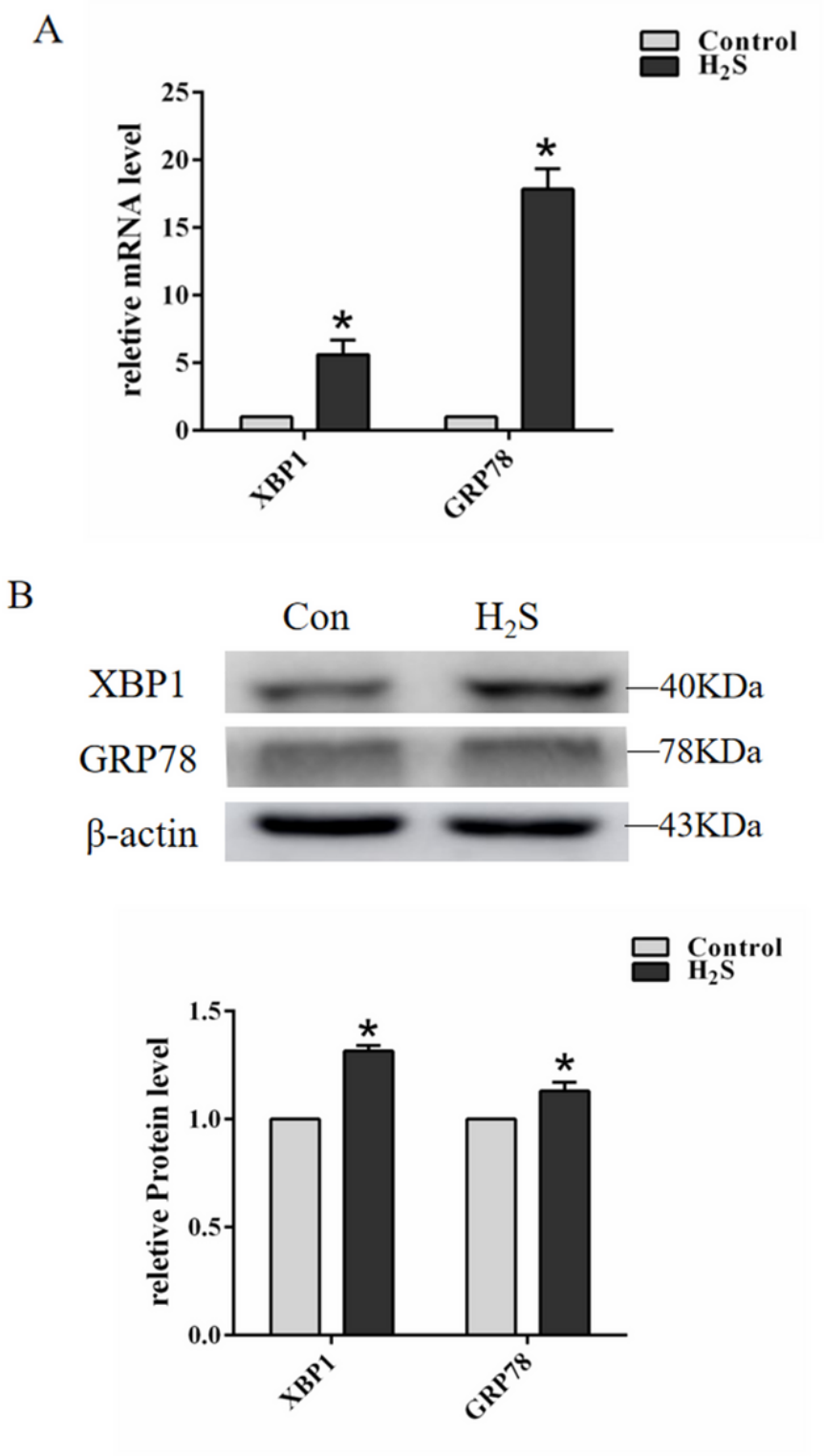

Figure 4

Expression levels of ER stress-related indicators in the jejunum of broilers exposed to H2S. (A) mRNA expression of ER stress-related indicators, including GRP78 and XBP1. (B) Relative protein levels of GRP78 and XBP1. β-actin was used as an internal reference in this experiment. All the values are expressed as the mean ± SD (n = 3). '*' above the bars denotes a significant difference ($P < 0.05$) between the control group and the H2S group.

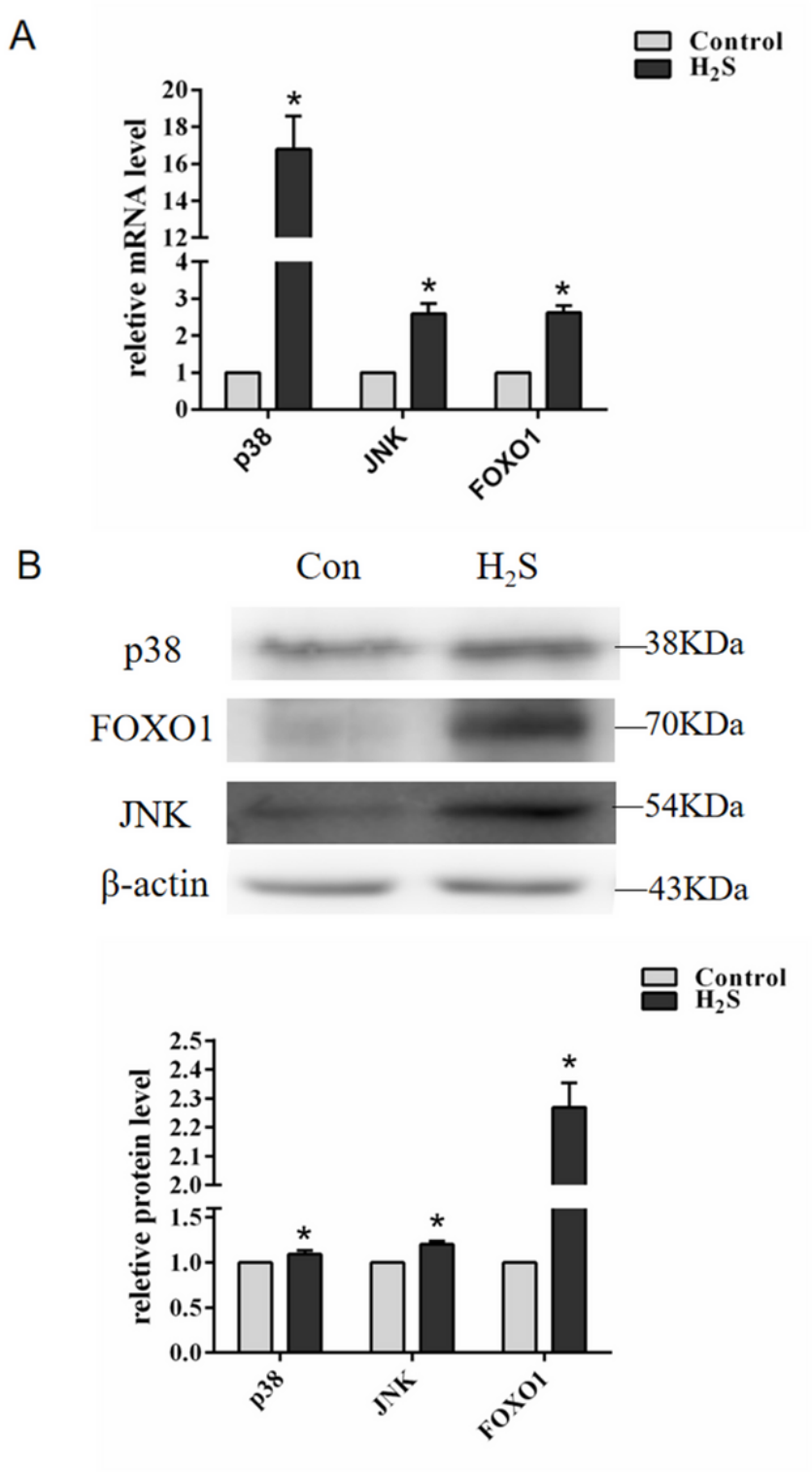

Figure 5

Expression levels of JNK, p38 and FOXO1 in the jejunum of broilers exposed to H2S. (A) mRNA levels of JNK, p38 and FOXO1, in the jejunum of broilers. (B) Effects of H2S on the protein expression levels of JNK, p38 and FOXO1 in the broiler jejunum. All values are expressed as the mean ± SD (n = 3). '*' above the bars denotes a significant difference ($P < 0.05$) between the control group and the H2S group.

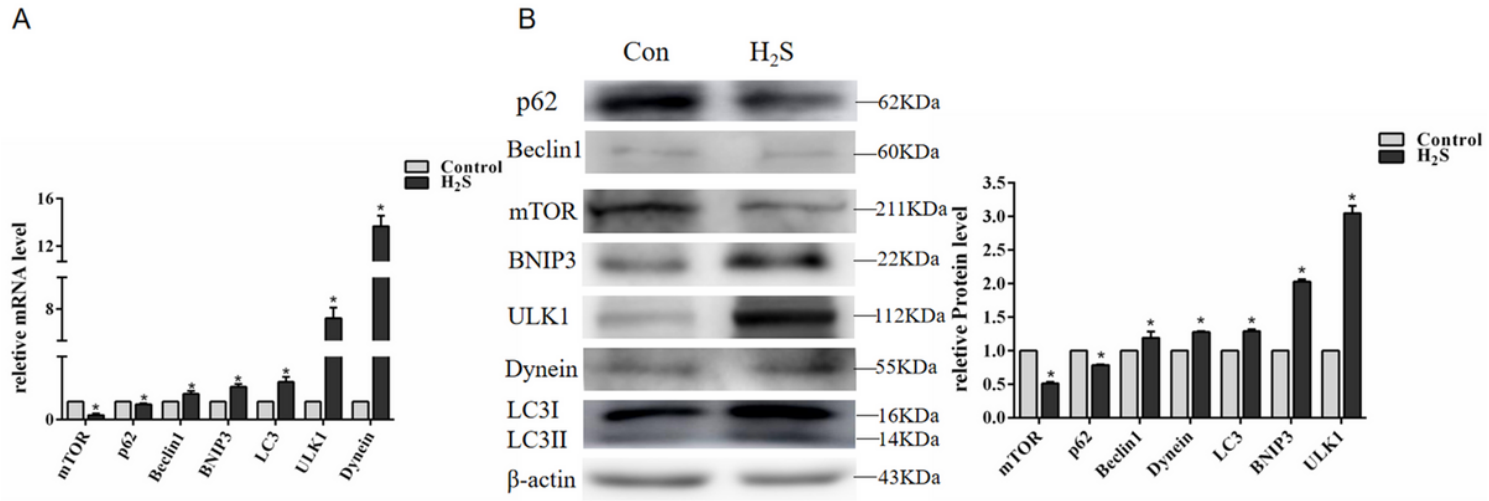

Figure 6

Expression levels of autophagy-related indicators in the jejunum of broilers exposed to H2S. (A) mRNA levels of Beclin1, mTOR, p62, Dynein, ULK1, BNIP3 and LC3 in the jejunum tissues. (B) Effects of H2S on the protein levels of autophagy-related indicators in the jejunum of broilers. β-actin was used as an internal reference. Data represent the mean ± SD (n = 3). '*' above the bars denotes a significant difference ($P < 0.05$) between the control group and the H2S group.

Supplementary Files

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- GraphicAbstract.tif
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